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Increased protein synthesis response to insulin in fibroblasts treated with the diacylglycerol kinase inhibitor R59022

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Insulin stimulated protein synthesis in quiescent 3T3 fibroblasts. This effect of the hormone was greater in the presence of the diacylglycerol kinase inhibitor R59022 (10^{-5} M) over a range of insulin concentrations from 1 μU to 1 mU/ml; R59022 increased the sensitivity of cells to insulin. The amount of radioactive diacylglycerol recovered from cells prelabelled with [^{3}H]glycerol was increased transiently in response to insulin; the response was larger and prolonged in cells given the kinase inhibitor. The results (i) support the hypothesis that diacylglycerol production is part of the signal pathway by which insulin stimulates protein synthesis and (ii) suggest that inhibition of diacylglycerol breakdown leads to increased sensitivity to the hormone.

Insulin; Diacylglycerol; Signal transduction; Protein synthesis; (Fibroblast)

1. INTRODUCTION

Stimulation of hormone and growth factor receptors leads to the generation of secondary signal molecules at the plasma membrane. One such mechanism is the hydrolysis, by a phospholipase C, of phospholipids to produce 1,2-diacylglycerol (DAG) and inositol phosphates [1]. The generation of endogenous DAG in turn then activates the key membrane enzyme protein kinase C [2,3]. Diacylglycerol is subsequently metabolised further by either a diacylglycerol kinase or a diacylglycerol lipase [4].

Insulin has effects on many biochemical reactions including a stimulation of overall cell protein synthesis [5,6]. The signal mechanisms by which insulin exerts these effects are only partly understood; insulin binds to a receptor in the cell plasma membrane and this leads to the generation and release from the membrane of one or more signal molecules [7,8]. Peptide mediators [9], glycophosphoinositides [10,11] and DAG [12] have been implicated in the control of fat and carbohydrate metabolism by insulin while phospholipase C activation, DAG and protein kinase C have been suggested to be involved in the signal mechanism whereby insulin stimulates protein synthesis [13,14].

The aim of the present work was to use an inhibitor of DAG kinase to investigate further the role of DAG as a signal in the sequence of events by which insulin stimulates protein synthesis in 3T3 fibroblasts.

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2. MATERIALS AND METHODS

The DAG kinase inhibitor R59022 (6-[2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl]-7-methyl-5H-thiazolo[3,2-α]pyrimidin-5-one) was purchased from Janssen Pharmaceuticals.

2.1. *Cell culture*

3T3 fibroblasts (Flow Labs, Irvine, Scotland) were grown in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 12% fetal calf serum (Gibco, England). After subculture cells were grown for 3–5 days and then 'stepped-down' by replacing the medium with DMEM containing only 4% serum; experiments were carried out 48 h later. Cells were grown either in 35-mm plastic petri dishes containing 2 ml medium (protein synthesis measurements) or in 100-mm dishes containing 8 ml

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medium (DAG production measurements). At subculture cells were seeded so as to achieve cell densities of 2–5 × 10^5 cells/35 mm dish and 16–30 × 10^5 cells/100 mm dish.

2.2. *Protein synthesis*

Protein synthesis was measured as in [13] by the incorporation of radiolabelled phenylalanine ([2,6-^{3}H]phenylalanine, 5 mCi/mmol, Amersham International, England) into cell protein; a high concentration of low specific activity radiolabelled precursor was used as in the flooding-dose method employed both in vivo and in isolated tissue [15,16]. Protein synthesis was measured over a 1 h period immediately after addition of insulin. The rate of protein synthesis was calculated from the phenylalanine incorporated per mg protein assuming that 4% of the cell protein is phenylalanine. The effects of insulin were calculated as percentage increases over the basal rate in control cultures given either no treatment or ethanol carrier.

2.3. *Diacylglycerol synthesis*

Cell glycerol-containing lipids were pre-labelled by incubating cells with 4 μCi [1,3-^{3}H]glycerol (3 mCi/mmol) for the 48 h immediately after they were put into medium containing only 4% serum. Lipid extraction was carried out using a modification of the method of Bligh and Dyer [17] and neutral lipids separated as described previously by thin-layer chromatography using LK5D silica gel plates (Whatman) with petroleum ether/diethyl ether/acetic acid (80:30:1) as the solvent phase [14,18]. Lipids were identified by co-migration with appropriate standards of 1,2- and 1,3-diacylglycerols, monoglyceride, triglyceride and free fatty acids. The radioactivity in DAG was expressed as a proportion of the total radioactivity recovered in cell lipids.

3. RESULTS

Addition of insulin to quiescent 3T3 cells led, as in previous experiments [13,14], to an increase in protein synthesis over the first hour after addition of hormone (table 1). Pre-incubation of cells with the DAG kinase inhibitor R59022 had no significant effect on the basal rate of protein synthesis; the mean inhibition from 8 experiments was 3.7% (table 1). However, in the presence of the inhibitor the stimulation of protein synthesis by insulin was considerably increased (table 1); in the presence of R59022 (10^{-5} M) insulin caused a 24% increase in protein synthesis compared to cells which had been treated with R59022 alone, whereas in control cells the mean stimulation by insulin was 12%. Thus, the response to insulin was doubled in these experiments.

Insulin stimulated protein synthesis in control cells at concentrations above 10 μU/ml but at 1 μU/ml there was no effect of the hormone (fig.1). However, in cells treated with the DAG kinase inhibitor insulin not only gave rise to greater stimulation of protein synthesis at all concentrations above 10 μU/ml but also produced marked stimulation at 1 and 10 μU/ml. The concentration dependence curve was shifted so that the sensitivity to insulin was increased; in control cells the insulin concentration for half-maximal response was approx. 50 μU/ml, and 5 μU/ml in cells treated with R59022.

Table 1

Effect of the diacylglycerol kinase inhibitor R59022 (10^{-5} M) on protein synthesis and its stimulation by insulin

	Ks (%/day)	Percentage stimulation by insulin
Control	48.2 ± 5.3 (8)	–
Insulin (1 mU/ml)	54.3 ± 5.5 (8)	11.8 ± 5.2 (8) (over control)
R59022 (10^{-5} M)	46.4 ± 6.1 (8)	–
R59022 + insulin	58.1 ± 8.2 (8)	23.6 ± 5.4[a] (8) (over R59022 only)

[a] $p < 0.05$ using a paired, 2-tailed 't'-test vs insulin alone

The effects of insulin (1 mU/ml) were studied in cells given 10^{-5} M R59022 or carrier only (ethanol) for 30 min prior to measurement of protein synthesis over 1 h. Results are presented as means ± SE, with the number of experiments using different cell preparations in parentheses

Diacylglycerol synthesis can be assessed qualitatively but not in absolute terms by measuring the amount of radioactivity in DAG after prelabelling of cells with [^{3}H]glycerol [19]. Using such methods R59022 alone was found to have no effect on DAG synthesis (as also found in [19,20]); in seven experiments the mean stimulation was 3 ± 9% (SE). However, in the presence of the inhibitor a marked stimulation (mean stimulation of 90 ± 16% using five separate cell preparations) of DAG labelling was detected in response to 1 mU/ml insulin (table 2). Insulin has been shown previously to stimulate DAG synthesis so that a transient increase in incorporation is observed 20–40 s after adding the hormone [14]. This effect was again noted in the present experiments (table 2) and moreover the amount of radioactivity recovered in DAG was increased and the peak of labelling was prolonged in the presence of R59022, as expected under conditions where there is effective inhibition of further metabolism of DAG (table 2). Lower

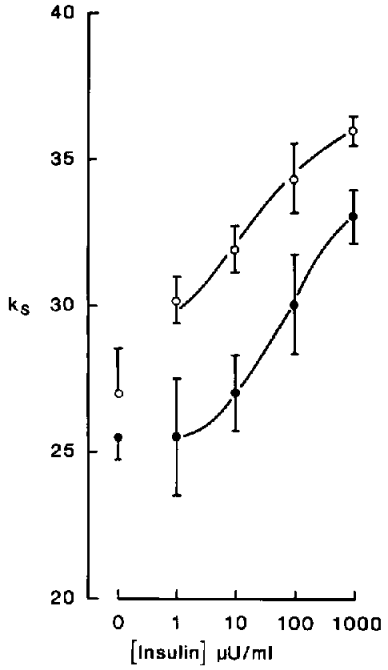

Fig.1. Sensitivity of protein synthesis to insulin and effect of the diacylglycerol kinase inhibitor R59022. Cells were pre-treated for 30 min with either 10^{-5} M R59022 (○) or ethanol carrier (●) and then given insulin. Protein synthesis was measured over the subsequent 1 h. Results shown are means from 4 or 5 determinations; error bars represent SE.

concentrations of insulin (10–100 μU/ml) were also effective in stimulating DAG synthesis and in the presence of R59022 it was possible to detect effects of 1 μU/ml insulin on DAG labelling (table 3).

Table 2

1,2-Diacylglycerol synthesis in response to insulin (1 mU/ml)

Time (s)	cpm recovered in DAG as % of those in total lipids	
	Control	+ R59022 (10^{-5} M)
0	0.57 ± 0.03 (9)	0.60 ± 0.15 (3)
20	0.70 ± 0.03 (3)[a]	0.69 ± 0.07 (3)
40	0.53 ± 0.02 (2)	0.76 ± 0.04 (3)[ab]
60	0.56 ± 0.05 (3)	0.72 ± 0.03 (3)[ab]
120	0.60 ± 0.03 (3)	0.69 ± 0.03 (3)[a]

[a] $p < 0.05$ vs control group at time zero
[b] $p < 0.05$ vs control group at corresponding time point using 2-tailed '*t*'-test

Cells were given R59022 (10^{-5} M) or ethanol carrier for 30 min prior to addition of insulin. 20–120 s after giving insulin lipids were extracted and radioactivity in the various fractions subsequently measured. Results are given as means ± SE with number of samples in parentheses

Table 3

Sensitivity of 1,2-diacylglycerol synthesis to insulin

[Insulin] (μU/ml)	cpm recovered in DAG 20 s after giving insulin (as % of cpm in total lipids)	
	Control	+ R59022 (10^{-5} M)
0	0.52 ± 0.03 (6)	N.D.
1	0.55 ± 0.01 (6)	0.69 ± 0.04 (3)[bc]
10	0.68 ± 0.05 (5)[b]	0.68 ± 0.04 (3)[b]
100	0.66 ± 0.04 (5)[a]	0.67 ± 0.03 (2)
1000	0.59 ± 0.02 (5)	N.D.

[a] $p < 0.02$, [b] $p < 0.01$ vs cells given no insulin
[c] $p < 0.02$ vs cells given 1 μU/ml insulin but no kinase inhibitor; both using 2-tailed '*t*'-test

Results are given as means ± SE with number of samples in parentheses. N.D., not determined

4. DISCUSSION

Inhibition of DAG kinase has been shown in both platelets and neutrophils to lead to increased DAG levels after stimulation by agonists which are thought to act through phospholipase C [20,21]. The present results show that in the presence of such a kinase inhibitor insulin causes an increase in the incorporation of radioactive glycerol into 1,2-DAG in 3T3 cells. After R59022 treatment insulin caused a larger and more prolonged increase in DAG labelling compared to the transient response found earlier with insulin alone [14]; this is consistent with a normally rapid turnover of DAG [4] which if blocked leads to increased amounts of the lipid in the membrane. Insulin concentrations of 10–100 μU/ml were found to stimulate DAG synthesis in control cells and in the presence of R59022 an effect of 1 μU/ml insulin was observed. Such concentrations (1–100 μU/ml) are well below those at which insulin binds to insulin-like growth factor receptors and therefore the effect on DAG synthesis most probably occurs through the insulin receptor itself. Together with earlier findings that insulin alone leads to increased DAG labelling [14,22] and to increased DAG production [23], these results support the hypothesis that insulin receptor activation leads to a stimulation of phospholipase C. The phospholipid

substrate from which this DAG arises remains, however, a matter for debate [8,22].

In addition to prolonging the increase in DAG labelling, R59022 treatment of cells also led to a greater response of protein synthesis to insulin and a greater sensitivity to insulin such that the concentration required for half-maximal stimulation was reduced. This increased stimulation of protein synthesis by insulin in the presence of R59022 suggests that potentiation of increased endogenous DAG levels leads to a greater protein synthesis response. In turn, this provides further evidence that DAG is a critical component of the signal pathway by which insulin stimulates protein synthesis and therefore supports the hypothesis [14] that this signal pathway involves a G-protein-dependent activation of phospholipase C to produce DAG which then activates protein kinase C. The present results also demonstrate that manipulation of membrane phospholipid metabolism so as to increase endogenous diacylglycerol in response to receptor activation provides an opportunity to increase the sensitivity of cell protein synthesis machinery to insulin. One would expect other responses to insulin which may possibly be controlled through DAG and protein kinase C, such as glucose transport [12,24], to be similarly affected.

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